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13. ABSTRACT *(Maximum 200 words)*

Corticotropin Releasing Hormone (CRF) is a neuropeptide, synthesized primarily by the paraventricular nucleus of the hypothalamus in the mammalian brain. CRF plays a central role in regulating the hypothalamic-pituitary-adrenal axis, both in the maintenance of homeostasis, and in the response to stress. The expression of CRF may be regulated at several levels, including gene transcription, mRNA stability, and translational and post-translational peptide processing and secretion.

Four independent lines of transgenic mice were made using 5 Kb of 5'-flanking control region of human CRF linked to a Neomycin Phosphotransferase II reporter. All four lines expressed the CRF transgene in hypothalamus, and three lines exhibited an appropriate induction in response to stress. These 3 lines were further examined, and exhibited regulation of the CRF transgene by the circadian rhythm, similar to the expression of endogenous CRF. This cycling of transgene expression was inhibited by dexamethasone, again similar to the well-characterized feedback regulation by glucocorticoids on endogenous CRF expression, a regulatory pattern which is restriced to the hypothalamus. In addition, male and female mice exhibited sexually dimorphic expression of the transgene, similar to that described and demonstrated for the endogenous CRF expression. It therefore appears that most, if not all, of the physiologic regulation of this complex CRF expression pattern is transcriptional.

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INTRODUCTION:

The Hypothalamic-Pituitary-Adrenal (HPA) axis plays an essential role in mammalian homeostasis, both in the regulated physiologic pathways associated with its diurnal cycles, and in the coordinated responses to stress. Corticotropin Releasing Factor (CRF), the hypothalamic neuropeptide hormone, acts as a potent ACTH secretagogue. CRF is the major regulator of ACTH release from the anterior pituitary, and thereby the resulting secretion of the adrenal glucoccorticoids, cortisol and corticosterone (1,2). Once released, these natural glucocorticoids inhibit the secretion and synthesis of both CRF and ACTH, via a negative feedback loop which defines this HPA axis (3,4). The biosynthetic and secretory status of CRF is central to this system. CRF release is influenced by a variety of endocrine and neural factors (5). These factors exert either a stimulatory or an inhibitory effect on CRF expression during various physiological states, including stress and the diurnal cycle (6, 7, 8).

Parvocellular neurosecretory neurons of the paraventricular nucleus of the hypothalamus (PVN) synthesize and secrete CRF. These neurons serve as the final common neuronal pathway, responding to and integrating the variety of stimuli and stressors that activate the HPA axis (1,2). In addition, the PVN contains additional neurosecretory cell types that can be recruited to supplement CRF secretion during stress (9). Hemorrhage, osmotic challenge, and adrenalectomy and subsequent corticosteroid withdrawal have all been shown to increase the levels of CRF mRNA and peptide (10,11,12,13).

Under normal conditions, many mammals, including mice and humans, exhibit a well-characterized diurnal pattern of CRF, ACTH, and adrenal steroid secretion (7). Often the steroid oscillations occur with the peak and nadir coinciding with the initiation and termination of the animal's activity period, respectively. The peak CRF level precedes the resulting increase in steroid, with a phase lead of about 2-3 hrs. (14). Various factors appear to affect the diurnal oscillation of the HPA components. The suprachiasmatic nucleus is thought to be important in generating the circadian cycle, as lesions made in this nucleus abolish the increases in ACTH and corticosterone (15). Catecholaminergic projections from the brainstem also appear to modulate the diurnal HPA rhythm, possibly via projections to CRF neurons (16). These projections have been proposed to exert a stimulatory influence on the hypothalamic CRF (17). The daily oscillation of steroid concentrations in plasma are important in maintaining the normal function of the HPA axis.

Gender based differences in basal and stress-induced HPA secretions in the rat were described as early as the 1960's (18) and there is evidence that these differences are promoted by gonadal steroid hormones at several levels of the HPA axis (19, 20). These changes are apparent at all levels of the axis, including higher levels of basal CRF in females (21). Nevertheless, the factors leading to sexually differentiated responses in HPA activity are relatively poorly defined.

CRF plays a central and early role in the HPA axis. Its expression varies and is regulated over relatively short time periods (1,2). In addition, modulation by glucocorticoids suggests transcriptional mechanisms participate, at least in feedback regulation of CRF. We therefore hypothesized that modulation of the HPA axis via CRF expression under other regulatory paradigms would also use transcriptional mechanisms. In this study we examined 4 lines of transgenic mice carrying a CRF transgene composed of the 5 Kb 5'-regulatory region of human CRF fused to the coding region of Neomycin phosphotransferase II. Evaluation of mRNA levels of the transgene does not reflect potential post-transcriptional regulation of CRF, including peptide processing (e.g. cleavage and amidation) or regulation of peptide secretion. We evaluated the expression pattern of the CRF transgene mRNA for circadian rhythmicity, glucocorticoid suppression of the circadian rhythm, the stress response, and sexual dimorphism. Based on these observations, we conclude that the regulatory promoter sequences of human CRF and their corresponding transcriptional regulatory mechanisms alone, are sufficient for the physiologic modulation of CRF gene expression in the PVN under each of these regulatory paradigms.

MATERIALS & METHODS

hCRF-Neo Transgenic Mice:

The human CRF genomic clone, SpHCRH-1 (22), was the generous gift of Shosaku Numa. The 5 kb upstream region was isolated as previously described (23) as a Eco RI-Xho I fragment using TthIII 1 partial digestion, ligation to an Xho I linker, and digestion with Eco RI. This CRF promoter was ligated to sequences encoding neomycin phosphotransferase II (Neo) (24) and SV40 splice and polyadenylation signals derived from the plasmid RSV Neo. The entire transgene containing the 5 kb CRF promoter, the 1 kb Neo coding sequence, and the 1.6 kb SV40 derived splice and polyadenylation sequences, was isolated as an Eco RI fragment. Injections were performed at DNX corporation (Princeton, NJ). Tail DNA preparations were analyzed by transfer to nylon membranes and hybridization to radioactive DNA probes derived from the 1 kb Bgl II-Sma I fragment of the Neo gene. Four transgenic founder animals were obtained, and transgenic lines were established by breeding to non-transgenic B6/SJL mice. F1, F2 and subsequent breedings to obtain homozygous mice were performed, and offspring were analyzed to confirm inheritance of the CRF-Neo transgenes.

Animal Procedures:

All the animals used in this study were housed in the Mouse Facility of the Division of Comparative Medicine, Washington University School of Medicine. The animal room was maintained on a 12/12h light/dark schedule (lights on at 06:30h) and housed to a maximum of 5 mice/cage. Food and water was made available ad libidum. A safe light was used to protect the animals from extraneous light sources.

Circadian Rhythm : Animals were acclimated and entrained in the light/dark cycle for at least 3 weeks prior to the start of the experiment. On the day of the experiment the animals (n=3) were sacrificed at 18:30h, 0:30h, 6:30h and 12:30h. The animals were euthanized and their brains and hypothalami were micro-dissected and frozen immediately. The time required for the sacrifice of all the animals at a single timepoint was no greater than 30 min.s.

Dexamethasone Suppression: The animals were injected with 2 mg/kg body wt. of dexamethasone IP at 10:00h on the day of sacrifice. Subsequent manipulations were similar to the control group as described above.

Stress Response: To observe the effect of stress, the animals were injected with 40 mg/kg body wt. of metapyrone (Sigma Chemical Co., St. Louis) IP in DMSO at 17:00h on the day prior to sacrifice. The animals were then further stressed (24) by housing them in the cold room (4 °C) with constant light overnight. The animals were then sacrificed at 15:00h the next day and their brains were dissected and frozen as described above.

Sexual Dimorphism: Male and female mice were sacrificed at 15:00h on the day of the experiment, and brains were dissected and frozen as described above.

Ribonuclease A/T1 Protection Assays:

The antisense neomycin phosphotransferase II (Neo) RNA was prepared from a pBKSII(-) Neo construct, containing the Neo gene (25) as a Bgl II - Sma I 1 kb fragment. The construct was linearized at the Nco I site and was used as template for T7 RNA polymerase which synthesized an antisense Neo RNA of 410 bp. The human gamma actin gene cloned in pGEM7Z, (a gift from C. Semenkovich, Washington University), was used as template for the actin control antisense RNA using T7 RNA polymerase. RNA probes were labeled using α-[^{32}P] CTP. Probes were then hybridized to 20 ug total RNA from the tissue and processed according to the manufacturer's specifications (RPA II Kit, Ambion Inc., Austin, TX). The reactions were then resolved by electrophoresis on 6% denaturing polyacrylamide gels, and exposed to storage screens overnight. End labeled DNA standards were used to confirm the size of the protected fragments.

Reverse-Transcriptase PCR:

RNA was isolated from hypothalamic tissue as described (26). Total RNA was then used to generate cDNA utilizing the following primers -

CRF:	Forward -	dCCAAGT-A/C-C-A/G-TTGAGAGACTGA
	Reverse -	dTTCCCCAGGCGGAGGAAGT
Cyclophilin:	Forward -	dTTCATCTGCACTGCCAAGAC
	Reverse -	dAACCCAAAGGGAACTGCAG

SuperScript reverse transcriptase (Life Technologies, Gaithersburg, MD) was used for cDNA synthesis as per manufacturer's instructions. As an internal control and for quantification, the subsequent PCR reaction was spiked with constant amount of plasmid DNA containing the complimentary sites for the two primers (27). The PCR product from the spiked plasmid DNA is of a different length when compared to that obtained from the cDNA. An external control was also performed using the cyclophilin transcript as the target sequence. The PCR reactions were performed for 25 cycles as follows:

CRF: 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 minutes.

Cyclophilin: 95 °C for 60 sec, 52 °C for 60 sec and 72 °C for 2 minutes.

The PCR reactions were then resolved on a 2% agarose gel.

Imaging and Quantitation:

The ribonuclease assays were visualized and quantified using a model 425 B PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant 2.0 software. The strength of the actin band and the Neo band were quantitated individually. The signal from actin mRNA was standardized arbitrarily to 1.0 and the same standardization factor was applied to the Neo signal. This value was assumed to represent the mRNA population. The image of the autoradiogram was processed for presentation using NIH Image 1.61 and Canvas 5.03.

The agarose gels containing the RT-PCR reactions were photographed with ethidium bromide UV fluorescence using Polaroid 557 film (Polaroid Corp.) The resulting negative was digitized on a Personal Densitometer (Molecular Dynamics) and quantitated using the ImageQuant software. A parallel experiment with known amounts of DNA was photographed and digitized for use as a standard.

RESULTS:

Regulated Hypothalamic Expression of the CRF-Neo Transgene:

One essential characteristic of CRF gene regulation is expression in the PVN and increased expression in response to stress (1,2). We wished to confirm that the CRF-Neo transgene, containing 5 kb of hCRF promoter sequence, displayed such an expression pattern, like that of the endogenous gene. We examined the CRF-Neo transgenic mice for 5 kb of the human CRF promoter hooked to the Neomycin gene. Appropriate targeting and expression of the CRF-Neo transgene in hypothalamus was confirmed using RT PCR (not shown) and RNase protection assays (Fig. 1). While each line, a result of an independent insertion event, exhibits quantitative differences in the levels of transgene expression, all four founder lines showed detectable basal expression of the CRF-Neo transgene in hypothalamus. Three of four lines showed increased expression of the transgene in response to stress (Fig. 1). These data indicate that the transgene contains information dictating appropriate targeting to the hypothalamus and the regulatory sequences necessary for a stress response.

hCRF-Neo transgene exhibits circadian rhythm:

The CRF mRNA displays a diurnal variation in its expression pattern (7). The mRNA oscillation occurs with the peak and the nadir often preceding the initiation and termination of the animal's activity periods. We picked 4 time points (n=3) to assay for Neo mRNA based on previous studies and in consideration of the light/dark cycle at the mouse housing facility. (Fig 2a). We limited all further experiments to the three transgenic lines that exhibited appropriate PVN stress regulation. The data points are duplicated to provide a visual representation of the circadian variation of the mRNA species. In accordance with previously published studies, we observed that mRNA from the transgene showed the appropriate diurnal rhythm. Transgene expression exhibited this diurnal change in expression level (amplitude) and the time the peak and nadir occurred (phase). The mRNA levels peak just before the onset of the activity period (afternoon) and drops to basal level before the resting period (dawn). As a control, we looked at the endogenous CRF mRNA levels using RT-PCR. Figure 2b shows the time points corresponding to the established peak and basal levels for mouse CRF expression. The data shows that the transgene, like the endogenous gene, is capable of responding to the clock signals that result in a diurnal expression profile.

Dexamethasone suppresses the circadian variations in the transgene:

Dexamethasone, when administered with the appropriate timing and dosage, blocks the circadian regulated expression of CRF mRNA (28). We tested the ability of the transgene to respond similarly to this feedback inhibition by corticosteroids. The experiment was performed similarly to previous determination of circadian rhythm, except in this case, the animals were injected with Dexamethasone IP at 17:00 hrs. on day 0, before the experiment. Both CRF Neo expression, and expression of the endogenous CRF mRNA were assayed by RT-PCR (Fig 2). As shown, the dexamethasone treatment represses the normal circadian peak for both endogenous CRF, and the CRF-Neo transgene message levels.

The Transgene exhibits Sexual Dimorphism:

Another aspect of CRF physiology is that in females, both the basal and stressed levels of CRF are elevated, relative to males (21). To evaluate this sexual dimorphism with the CRF-Neo transgenic mice, we used RNase Protection Assays and RT-PCR of both the CRF-Neo transgene and the endogenous CRF gene to compare males and females. As shown in Figure 3, for all the transgenic lines, females have elevated basal and stress levels of both CRF-Neo transgene and endogenous CRF expression. Thus the 5 kb CRF-Neo transgene is sufficient to impart sexually dimorphic CRF expression.

CONCLUSION:

The present study used transgenic mice to evaluate several well-established regulatory features of CRF physiology, including targeting to the PVN, the stress response (which results in increased expression), regulation by circadian rhythms (resulting in diurnal variation), feedback regulation by glucocorticoids (resulting in suppression of expression without diurnal variation), and sexual dimorphism of CRF gene expression (resulting in higher expression in females). In a previous study, Keegan et. al., used a rat CRF promoter to drive CAT and β-gal reporter constructs to observe the cell specific expression pattern during development (29). The present study used a CRF-Neo fusion reporter gene driven by the hCRF promoter (30). By determining the levels of the reporter mRNA, we were able to focus exclusively on the contribution of transcriptional mechanisms to CRF biology, independent of the potential contributions of post-transcriptional mechanisms, secretory control, and regulation or processing of the CRF peptide itself. For each of the four different CRF regulatory paradigms examined, the regulation of the transgene was indistinguishable from that of the endogenous CRF gene. It should be further noted that the CRF-Neo transgene contained only the 5 kb upstream regulatory region. No other DNA sequences from the CRF gene with potential regulatory functions were present, including the untranslated sequences in exon 1, the intron, the coding region in exon 2, and untranslated sequences and polyadenylation sites in the 3'-end of the gene. Thus all of the regulation of CRF transgene expression observed was transcriptional, and the 5 kb upstream region alone, without contributions from other regions of the gene, was sufficient to transfer this regulation to the Neo reporter sequences.

The diurnal variation observed in both the CRF message and peptide is thought to be affected by neuronal projections from the SCN, the major mammalian circadian pacemaker (16, 17). In the superchiasmatic nucleus, the mammalian circadian pacemaker, transcriptional activity varies with the diurnal cycle. In addition, *sim* and *clock* proteins with transcriptional activity (31) have recently been identified as participating in the activity of circadian pacemakers. In our studies, the diurnal rhythm of gene expression was transferred from CRF to the Neo reporter gene by the CRF cis-acting regulatory sequence. This argues that transcriptional mechanisms also participate in the circadian pattern of CRF oscillations.

Feedback regulation by glucocorticoids is an essential and defining characteristic of hypothalamic CRF expression. Only the CRF expressed in the PVN is responsive to glucocorticoid feedback, while the CRF expressed in other sites in the brain, peripheral organs and placenta is not (32, 33). Therefore, this characteristic regulation pattern is not only an indicator of appropriate physiologic regulation of the transgene, but also serves as additional confirmation that transgene expression is targeted to the correct neuronal cell type. Our observation that dexamethasone suppression of the circadian oscillations of CRF mRNA was transferred to the CRF-Neo mRNA of the transgene, demonstrates that this feature of CRF expression is fully transcriptionally regulated and that this regulation is mediated by the 5'-flanking control region, without a requirement for other regulatory regions. Regulation of gene expression by glucocorticoids is typically mediated by the glucocorticoid receptor (34), a well-characterized transcription factor. However, studies to date have been unable to find the appropriate GRE recognition sequences in the CRF promoter to account for this regulation. This suggests the consideration of regulatory mechanisms without a requirement for GR DNA binding sites in the CRF regulatory region. One possibility is protein-protein interaction of GR with another transcription factor participating in CRF regulation within the PVN neurons that express CRF. Another possibility would be that glucocorticoid regulation occurs, not by either direct or indirect interaction between the CRF promoter and GR in the CRF neurons, but by GR mediated regulation at other neural sites, e.g. hippocampal neurons, which have projections to the PVN. While the data presented demonstrates that glucocorticoid regulation of CRF is transcriptional, these mechanistic details remain to be evaluated in future studies.

The sexually dimorphic expression pattern of CRF indicates a role for circulating sex steroids and their nuclear receptors in CRF regulation (19,20). As described for regulation by glucocorticoids, the sex steroids typically exert their effects via specific nuclear receptors that act as transcription factors (35). There have been reports of direct transcriptional regulation of CRF by estrogen receptor via interaction with DNA sequences in the CRF promoter (36). We recently re-examined regulation of CRF by ER, and found no evidence for direct transcriptional regulation by ER via binding to CRF sequences (37). As described above for glucocorticoid regulation, one may therefore consider regulatory mechanisms without a requirement for direct ER binding to CRF DNA sequences. For estrogenic regulation, in addition to protein-protein interactions within the PVN neurons, or ER mediated regulation at other neural sites, one must now also consider another possibility. Recently a new form of ER, ER beta, has been described (38). ER beta has a wide distribution in the CNS (39), and ER beta is now also a candidate as a participant in the sexually dimorphic CRF expression, either via effects within the PVN neurons, or via projections from other neurons.

The characteristic increase in CRF message and peptide under conditions of stress also have suggested regulation by multiple transcriptional interactions. Stress has been shown to increase immunoreactive Fos and Jun in the PVN (40). Stress also increases the plasma levels of corticosteroids (1,2), and hence, activated glucocorticoid receptor, also suggesting transcriptional mechanisms. In addition, the CRF promoter has a functional CRE sequence, suggesting cAMP pathways leading to CREB may also participate in transcriptional regulation of CRF under the stress paradigm (41). Fos activity has been shown to be influenced by a wide variety of stimuli in many sites in the brain (42), and there is evidence that increases in fos expression can be regulated by neuronal inputs from other centers (43). The role of any or all of these transcription factors in CRF regulation and the mechanistic details of the stress response remain for future studies. However, the regulation of the expression of transcription factors by neuronal signals, using Fos as a model, is likely to participate in the changes in CRF transcription.

As discussed above, each of the regulatory paradigms evaluated in our studies had at least some evidence to suggest that transcriptional mechanisms would participate in CRF expression (44). Yet, these studies demonstrated some features of CRF regulation that were not as expected. Chief among these is that the 5'-flanking region of CRF was sufficient, not only for targeting to the PVN, but also for the transfer of full and appropriate physiological regulation to a heterologous reporter. DNA sequences form exons, introns, and 3'-flanking regions of the gene were not required. These results complement previous studies of CRF expression in transgenic mice using the metallothionein promoter to express the CRF DNA sequences 3'- to the transcription start site including the peptide coding region (45). That study found that CRF sequences restricted the expression of the metallothionein promoter, and suggested that CRF 3'-sequences do carry signals contributing to gene targeting and expression. Our results indicate that the contributions of these 3'-signals to targeting are not absolutely required when 5'-CRF sequences are present. Their contribution may still be important, and we cannot exclude the possibility that the absence of these signals was responsible for the failure of one of our four founder lines to regulate appropriately.

The completeness of the regulatory control exhibited by the CRF-Neo transgene is also somewhat unexpected. The CRF peptide has pre- and pro-peptide leaders responsible for the secretion and appropriate processing of CRF in the PVN neurons. The authentic CRF peptide is amidated prior to its secretion into the hypophysial portal system (46). There is also evidence that CRF is stored in neuronal vesicles, and that acute stimuli cause release of stored CRF, as well as synthesis of new hormone (47).

All of these processing steps are points for potential modulation of expression of CRF at the post-translational level. While each of these mechanisms may also be regulated, the data presented above indicate that these peptide processing steps, even though required for the activity of the CRF peptide and its appropriate delivery to the hypophyseal-portal circulation, are nonetheless, secondary in importance to transcriptional mechanisms when considering the changes in activity of CRF in reponse to the physiologic signals. The CRF-Neo transgene used the 3'-end splice and polyadenylation signals derived from SV40 which are often used to allow mRNA processing of expressed cDNA's. Again, the use of this heterologous 3'-end, rather than the endogenous exon 1 to exon 2 splice site and CRF 3'-polyadenylation signal also excludes a requirement for regulation of splicing, or for regulatory mechanisms encoded in the 3'-ends of some mRNA's, such as signals regulating mRNA degradation,

The regulation and physiology of CRF is complex, requiring the appropriate responses to an abundance of regulatory signals. Surprisingly, it appears that most, if not all, of the important regulation of this gene is transcriptional. Furthermore, the 5'-flanking region of the gene contains regulatory code sufficient not only to target gene expression to the appropriate neuronal nucleus, but also to regulate even a heterologous gene, exclusive of other DNA signals. These data support the notion that a virtually complete understanding of the regulation of CRF and its physiology might be obtained from a detailed understanding of the cellular transcription factors in the PVN neurons, their interactions with each other, and with their DNA regulatory sequences in the 5'-flanking control region of the CRF gene.

REFERENCES: None.

FIGURE LEGENDS:

Figure 1:

RNase Protection Assay of Total Hypothalamic RNA from normal and stressed transgenic mice.

(A): RNase Protection Assay was performed on total RNA isolated from the hypothalamic region of the mice from the 4 transgenic lines. Antisense NPTII RNA (Neo) spanning the coding region was used as probe. Antisense Human γ-Actin was used as control for amount of total RNA in the reactions. The gels were then exposed on a PhosphorImager from Molecular Dynamics.

(B): The bands in (A) were quantitated on the PhosphorImager using ImageQuant. The values for the Neo band were standardized using those for the γ-Actin and the resulting numbers were plotted on a histogram with the amount of Neo set arbitrarily as 1.00.

Figure2:

The Neo Transgene reflects endogenous circadian rhythms.

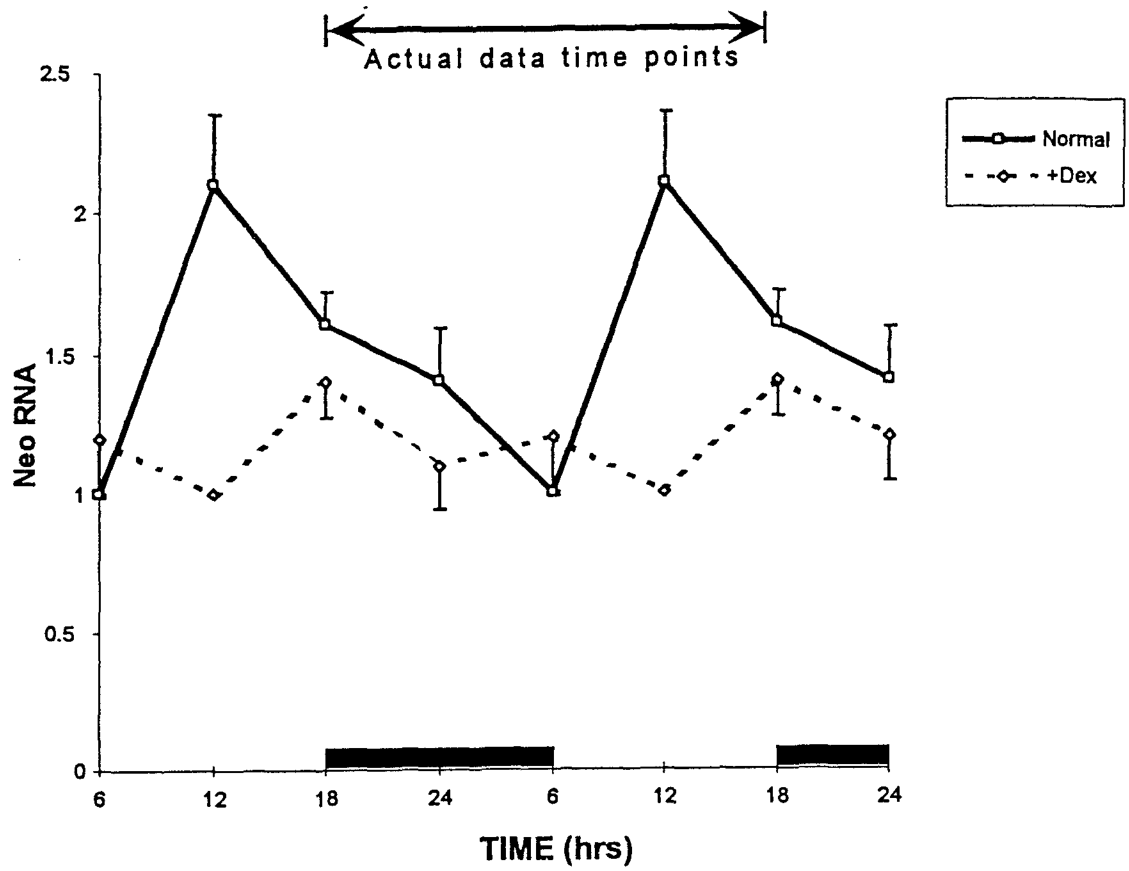
(A): The Neo transgene (solid line) was quantitated using RPA at 4 different time points and the data from all 3 lines were combined (n=9). A similar assay was performed in mice treated with dexamethasone (dotted line).

(B): The endogenous CRF was assayed using RT-PCR from both untreated and dexamethasone treated mice. Only 2 time points corresponding to the zenith and nadir of the expected RNA levels were picked for the assay. The RNA level at the nadir was arbitrarily set to 1.00.

Figure3:

The Neo Transgene shows sexual dimorphism.

(A): The Neo transgene was quantitated using RPA, from the males and females of each transgenic line (n=3). The RNA levels from the males were arbitrarily set to 1.00.
(B): A similar experiment was performed on the endogenous CRF, which was assayed using RT-PCR.



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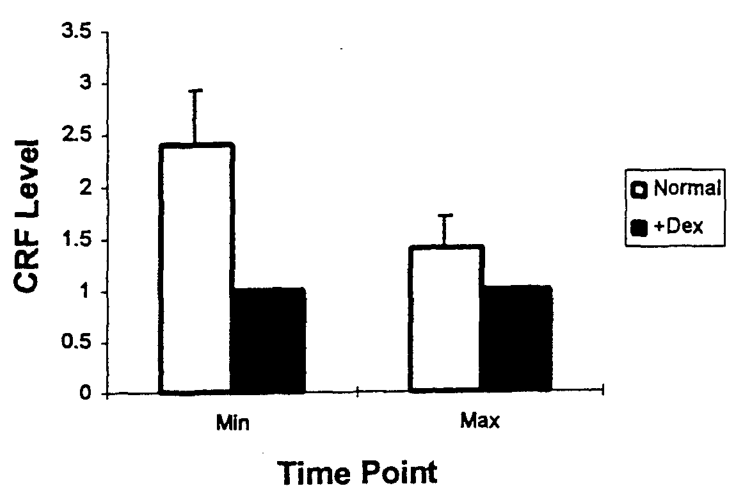

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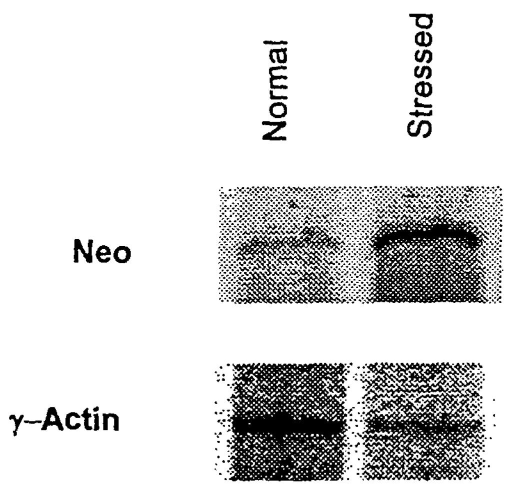
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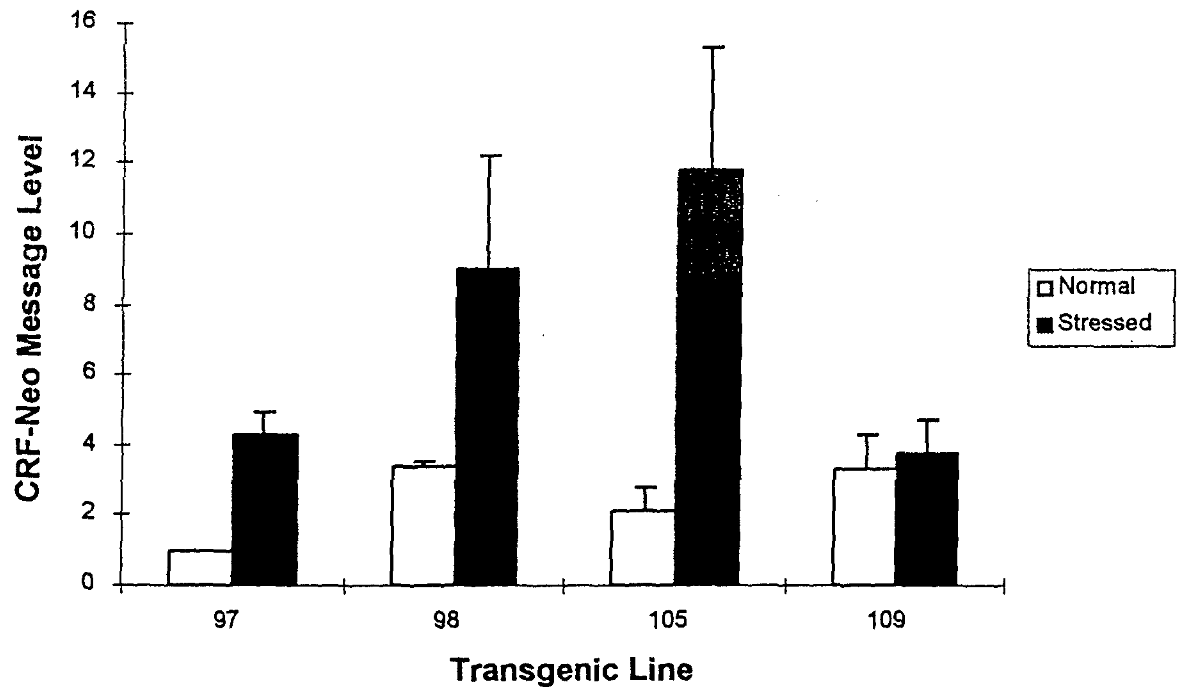
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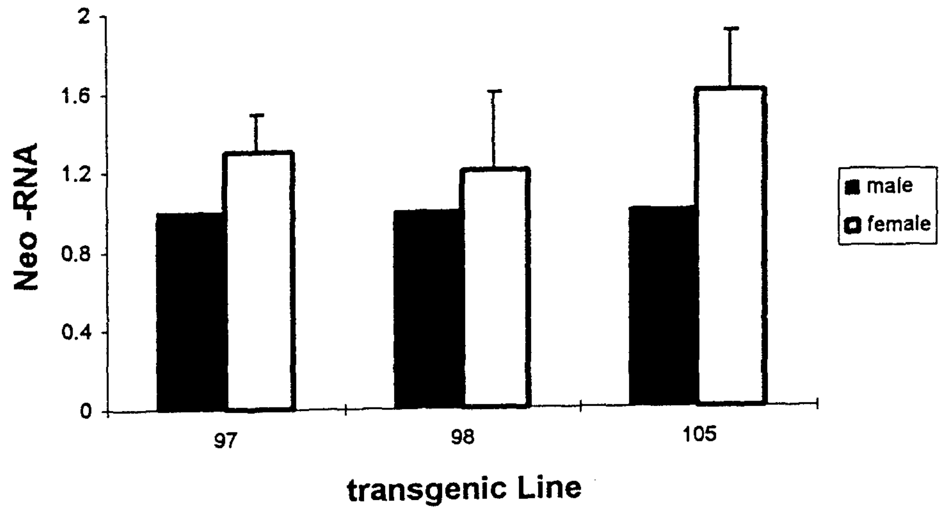
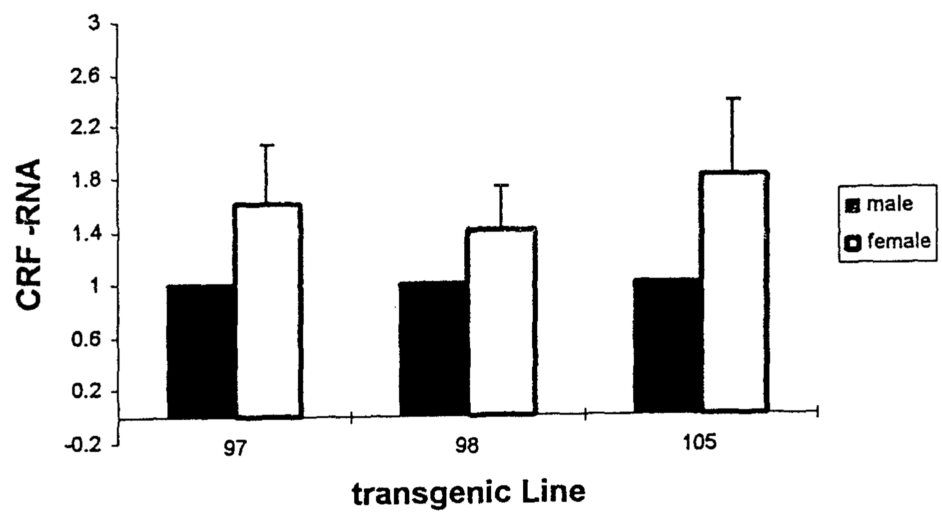
Sexual Dimorphism





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